Original Article: Evaluation of miR137 gene expression on the survival rate of patients undergoing colorectal surgery with a history of neoadjuvant therapy

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ABSTRACT

Introduction: More than 400 colorectal tissues, including colorectal adenomas and cancers, and a panel of six CRC cell lines were used to study the epigenetic regulation of miR-137. **Material and Methods:** We go over miR-137's epigenetic control and how it affects the development of colorectal cancer. Six CRC cell lines, 50 colorectal tissues, 21 healthy individuals' normal colonic mucosa (N-N), 160 primary CRC tissues, and their corresponding normal mucosa (N-C), as well as 68 adenomas, were used to determine the methylation status of the miR-137 CpG island. We examined the expression of miR-137 using TaqMan RT-PCR and in situ hybridization. **Results:** MiR-137 was only expressed in colonic epithelial cells, which cover the entire colonic crypt, in normal colonic mucosa. However, none of the adenomatous and CRC samples exhibited miR-137 expression, supporting our finding that miR-137 is silenced in the majority of colonic neoplastic tissues. As a result of our discovery that CpG island methylation causes miR-137 to be epigenetically silenced in CRC, we then carried out functional studies to see if miR-137 had tumor-suppressive properties in vitro after transfecting CRC cell lines with miR-137 precursor. **Conclusion:** In conclusion, this study first explains that miR-137 acts as a tumor suppressor in the colon, is frequently silenced in CRC through promoter hypermethylation, and its restoration inhibits cell proliferation in vitro.

Introduction

The lifetime probabilities of developing invasive CRC in the US are 5% in males (1 in 20) and 4% in females (1 in 22). The median age at diagnosis is approximately 70 years. Colorectal cancer (CRC) is the third most common cancer in both men and women, accounts for 8% of new cancer cases in the United States (US), and is responsible for 8% to 9% of the estimated cancer deaths in the US in 2014 dot. The incidence rate of CRC varies greatly between regions of the world; it is ten times higher in the US and Europe than in Africa

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and Asia [1-3]. First-generation immigrants have the incidence rates of their native country, whereas incidence rates for second-generation immigrants adjust to those of the country of immigration [4].

Red meat (beef and pork), alcohol use, and obesity are known risk factors for CRC in the Western lifestyle and are linked to a higher risk of the disease. Additionally, patients with inflammatory bowel disease, such as Crohn's disease and ulcerative colitis, have a higher risk of developing CRC and should be closely monitored. Five percent of cases of CRC are thought to be caused by hereditary syndromes like familial adenomatous polyposis (FAP) and hereditary non-polyposis CRC (HPNNC) [5-7].

Another 20% of cases are thought to be explained by familial clustering. The vast majority (roughly 75%) of CRC cases are sporadic. The stage of CRC affects the likelihood of recovery and survival. The primary tumor's size (T stage), the lymph nodes' involvement (N stage), and the presence of distant metastases are used to stage the condition. MicroRNAs (miRNAs) play a role in the pathogenesis of numerous cancers, including CRC [8-10].

MiRNAs may function as tumor suppressor genes (tsmiRs) or oncogenes (oncomiRs), according to mounting evidence. They may also play a role in the early stages of carcinogenesis. MiRNA expression profiles can have prognostic and therapeutic implications, and they can be used to classify various types and subtypes of cancer based on their miRNA expression patterns. In contrast to mRNA, a small number of miRNAs may be adequate for clinical uses, and more intriguingly, miRNAs are largely unaffected by RNA degradation and remain mostly intact in various tissues [11-13].

MiRNAs are an extremely exciting and promising tool for early tumor detection, prognostication, and treatment because of all these characteristics. It is still unknown what causes the miRNAs to express differently in tumor and normal cells on such a large scale. About 20% of all miRNAs are located within CpG islands, and pharmacological unmasking of silenced miRNAs using epigenetic drugs has shown that several miRNAs can be inactivated by this mechanism in CRC cell lines [14-16].

These studies have made it possible to identify the miRNAs that are methylation-silenced in CRC patients, but the idea of miRNA epigenetic regulation in the development of colorectal cancer is still largely unexplored. One such miRNA is miR-137, which can be found on chromosome 1p22 inside the non-protein coding RNA gene AK094607 [17-19].

In many tumors, methylation frequently silences this miRNA, which is encased in a CpG island. Ectopic transfection of the miR-137 precursor in oral cancer and glioblastoma multiforme inhibited cell growth, pointing to its tumor suppressive activity. The biological function of miR-137 and its specific downstream mRNA targets in the development of colorectal cancer are still unknown. Furthermore, virtually no information exists regarding miR-137 disruption in adenomas, the precursor lesion of CRC. By addressing some of the above-mentioned problems, we have for the first time comprehensively characterized the function of miR-137 in the colon [20-22].

More than 400 colorectal tissues, including colorectal adenomas and cancers, and a panel of six CRC cell lines were used to study the epigenetic regulation of miR-137. Through in vitro tumor suppression research and whole genome expression profiling, we were able to pinpoint potential mRNA targets for miR-137. LSD1, a crucial component of the epigenetic apparatus, has also been successfully validated as one of miR-137's targets [23-25].

Material and Methods

Cell lines and 5-aza-2-deoxy-cytidine treatment

Six different CRC cell lines—HCT116, LoVo, RKO, SW48, HT29, and SW480—that we acquired from the American Type Culture Collection (ATCC, Manassas, VA) over the previous two years were used in this study. All cells in our lab are examined and verified every six months using well-known genetic and epigenetic markers. The proper culture conditions were used to grow the cells. For demethylation tests, cells were exposed to 2-point5 umol/L 5-aza-2-deoxy-cytidine (5-AZA; Sigma) for 72 hours, with the medium and medication being changed every 24 hours.

Tissue specimens

In this study, 50 colorectal tissues were examined, including 14 sporadic primary CRCs with their corresponding adjacent normal colonic mucosa (N-C) and 68 colorectal adenomas obtained from the Okayama University Hospital, Okayama, Japan. The Hospital Clinic of Barcelona, Spain, collected 21 samples of normal colonic mucosa from non-tumor patients (N-N). At the Baylor University Medical Center in Dallas, Texas, 47 CRC tissues and the corresponding adjacent normal mucosa from 11 patients with Lynch syndrome, 14 patients with microsatellite-unstable CRCs, and 22 patients with microsatellite-stable tumors were also collected. The Institutional Review Boards of each participating institution approved the study after receiving the written informed consent from each patient.

RNA extraction and DNA methylation analysis

According to the manufacturer's instructions, total RNA extraction was performed using the miRVana RNA extraction kit and the RecoverAll kit, both from Ambion Inc in Austin, Texas. Using three different methods, PCR analysis of bisulfite-modified genomic DNA (EZ DNA methylation Gold Kit, Zymo Research) was used to determine the DNA methylation status of the miR-137 CpG island. Bidirectional bisulfite sequencing was used to examine the methylation status of the HCT116 and RKO cell lines. Second, using primers for either the methylated or unmethylated DNA in the six CRC cell lines used in the study, we carried out methylation specific PCR (MSP). Quantitative methylation analysis was performed using the PSQ HS 96A pyrosequencing system from QIAGEN using bisulfite pyrosequencing.

Analysis of miRNA expression using TaqMan RT-PCR

The TaqMan miRNA Assay (Applied Biosystems Inc.) was used to examine the expression of mature miR-137. Foster City, California). The expression of RNU6B (Applied Biosystems Inc, Foster City, CA) was utilized as an endogenous control. Each experiment was carried out in triplicate.

In situ hybridization

On 5-mm FFPE sections taken from five normal colonic mucosa tissues, three adenomatous polyps, and five colorectal adenocarcinomas, miR-137 was in situ detected. Briefly, the slides were hybridized with 10 pmol probe (LNA-modified and DIG-labeled oligonucleotide; Exiqon) complementary to miR-137 and after incubation with anti-DIG-AP Fab fragments conjugated to alkaline phosphatase, and the hybridized probes were detected by applying nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate color substrate (Roche) to the slides. For every hybridization step, there were positive controls (RNU6B, Exiqon) and no probe controls.

Transfection of miR-137 precursor molecules

Pre-miR miRNA precursor molecules (Ambion Inc., Austin, TX) or Pre-miR miRNA negative control 1 (Ambion Inc., Austin, TX) were transfected into HCT116 cells using Lipofectamine 2000 (Invitrogen, Rockville, MD) in accordance with the manufacturer's recommendations. Total RNA was extracted 48 hours after transfection for RT-PCR and microarray analysis, and cell lysates were made 48 hours later for Western blot analysis. We used Western blotting to confirm the protein downregulation of CDK6, a previously validated target, in order to ensure the transfection efficiency.

Gene expression microarray analysis, RT-PCR and miRNA target prediction

Control miRNA precursor or miR-137 precursor was transfected into HCT116 cells as previously mentioned. Illumina's TotalPrep RNA Amplification Kit was used to amp up the extracted RNA. The Agilent 2100 Bioanalyzer was used to evaluate RNA integrity. Labeled cRNA was hybridized overnight to Human HT-12 V3 chips, then washed and scanned on an Illumina BeadStation-500. The signal intensity values from the scans were processed using Illumina's BeadStudio version 3.1 and background subtracted. The Lumi R-package was used for normalization, and quantiles were used. Calculations of fold-changes were made in

relation to each control. The miRecords website (http://mirecords.MiR-137's miRNA targeting was predicted using data from umn .edu/miRecords)(15).

Genes that were downregulated (>2 fold-change) after miR-137 precursor transfection in the microarray were crossed with the genes predicted to be targets based on miRecords in order to reduce the list of predicted targets. For validation, genes that had been previously linked to either carcinogenesis generally or CRC specifically were chosen. The Advantage RT-for PCR Kit (Clontech Laboratories, CA) and random hexamers were used to reverse transcribe 1 g of total RNA into cDNA for reverse transcription-PCR (RT-PCR). Power SYBR Green (Applied Biosystems, Inc, Foster City, CA) RT-PCR was conducted for a subset of the targets identified using the aforementioned method. Results were adjusted to account for -actin expression. There were three duplicates of each experiment.

Western blot analysis

Standard procedures were used to perform the Western blot analysis. The primary antibodies used were anti-CDK6 (Cell Signaling, MA), anti-LSD1 (Cell Signaling, MA), anti-SEMA4D (BD Transduction Laboratories, San Jose, CA), anti-AURKA (Cell Signaling, MA), anti-BX1 (Cell Signaling, MA), and anti-actin antibody (Clone AC-15) at 1:32000 dilution.

Luciferase reporter assay

The pMIR Reporter Luciferase vector (Ambion Inc, Austin, TX) was used to create luciferase constructs by ligating oligonucleotides containing the wild-type or mutant putative target site of the LSD1 3′-UTR downstream of the luciferase gene. Using Lipofectamine 2000 (Invitrogen, Rockville, MD), cells were co-transfected with 400 ng of a firefly luciferase reporter vector containing either wild-type or mutant oligonucleotides, 200 ng of a pGal control vector, and 50 pmol of either a miR-137 precursor or a negative control. As a control, the parental luciferase plasmid was also transfected. Beta-galactosidase Enzyme Assay System from Promega was used to normalize the measurement of luciferase activity 48 hours after transfection. In three different experiments, experiments were carried out in triplicate.

Bromodeoxyuridine (BrdU) proliferation assay

The proliferation index was determined by BrDU incorporation in colon cancer cells 96 hours after transfection of either a control miRNA precursor or a miR-137 precursor as described above (Cell Proliferation ELISA, BrdU, Roche), in accordance with the manufacturer's instructions. Three separate experiments were carried out in triplicate.

Statistical analysis

Graph Pad Prism 4.0 (San Diego, CA) and SPSS 13 (Chicago, IL, USA) statistical software were used to analyze all of the data. The Student's test, Wilcoxon test (non-parametric paired analysis), and Mann-Whitney U test (non-paired analysis) were used to analyze quantitative variables. Depending on the situation, the Fisher's test or the Chi sq test was used to analyze qualitative variables, a p-value with two sides.

Ethical approve

This study was carried out after being approved by the Ethics Committee of Tabriz University of Medical Sciences (No: IR.TBZMED.REC.1398. 801).

Results

We employed a sequential methodology to assess the DNA methylation status in the miR-137 CpG island. Figure 1 shows the miR-137 CpG island and the locations of the PCR products generated by each method. First, we used direct bisulfite sequencing to examine the promoter region of miR-137 in the HCT116 and RKO cell lines. We discovered that both cell lines had extensive methylation throughout this region, which was then reversed after 5-AZA treatment . Second, we discovered using MSP that the miR-137 CpG island was heavily methylated in all of the CRC cell lines, and as predicted, treatment with 5-AZA induced significant demethylation The third step was to use quantitative pyrosequencing to analyze the methylation of the miR-137 CpG island.

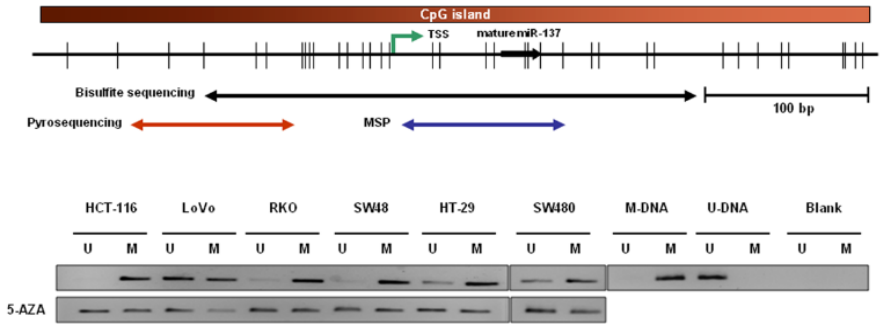

Figure 1: miR-137 CpG island and the locations of the PCR products generated by each method

The level of miR-137 expression was then examined using TaqMan RT-PCR in the same panel of CRC cell lines, and we discovered an inverse relationship between the degree of CpG island methylation as determined by pyrosequencing and the level of expression (Figure 2). Treatment with 5-AZA resulted in demethylation as well as upregulation of miR-137 in CRC cell lines, indicating that most CRC cell lines have CpG island promoter methylation that inhibits miR-137 expression.

Next, we examined the methylation status in a cohort of colorectal cancer tissues using the miR-137 pyrosequencing assay, which included 68 colorectal adenomas, 113 CRC tissues, and 21 normal mucosa from non-tumor patients (N-N). Finally, and perhaps most intriguingly, miR-137 methylation in adenomatous tissues was found to be at the same level as that in CRC tissues (p=0.8352), indicating that methylation of this miRNA is a precursor to colorectal carcinogenesis. We then looked at the miR-137 methylation status in tumor and corresponding normal tissues (N-C) from 11 patients with Lynch syndrome, 14 patients with sporadic MSI, and 22 MSS patients to see if there might be a difference between the various molecular subtypes of CRC based on the presence of microsatellite instability (MSI). Lynch, sporadic MSI, and MSS had mean methylation levels of 22 percent, 27 point 3 percent, and 24 point 7 percent, respectively. The corresponding percentages in N-C were 6 point 4 percent, 6 point 2 percent, and 7 point 3 percent, respectively. As a result, miR-137 methylation appears to be present in all molecular subtypes of CRC.

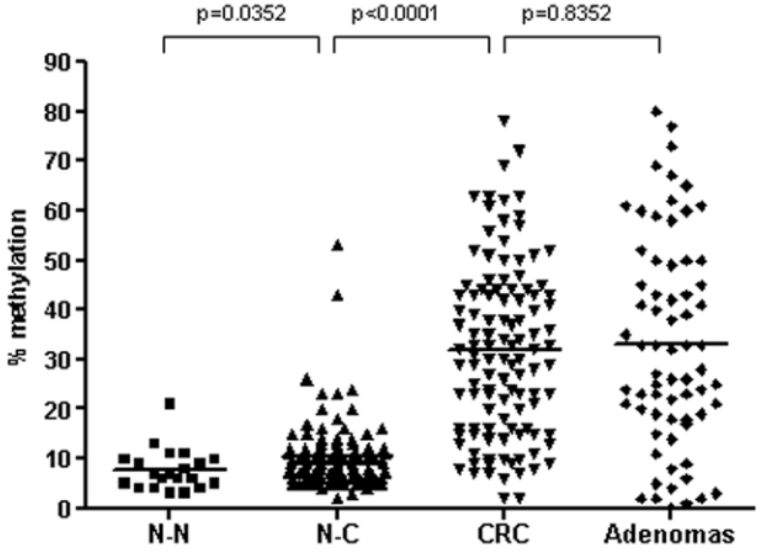

Figure 2: miR-137 CpG island in CRC cell lines and in CRC tissues

We examined the expression of miR-137 using TaqMan RT-PCR in 15 pairs of CRC and healthy colonic mucosa, and we discovered that the expression was significantly downregulated in the tumor when compared to the healthy mucosa (Figure 3). It's interesting to note that the only 2 cases (data not shown) without methylation did not exhibit downregulation in the tumor tissue. We carried out in situ hybridization using 5′-DIG-labeled LNA probes, a method that has been used to detect miRNA in situ in FFPE tissues, to find out which particular cell types expressed miR-137 in the colon (Figure 3). MiR-137 was only expressed in colonic epithelial cells, which cover the entire colonic crypt, in normal colonic mucosa. However, none of the adenomatous and CRC samples exhibited miR-137 expression, supporting our finding that miR-137 is silenced in the majority of colonic neoplastic tissues. As a result of our discovery that CpG island methylation causes miR-137 to be epigenetically silenced in CRC, we then carried out functional studies to see if miR-137 had tumor-suppressive properties in vitro after transfecting CRC cell lines with miR-137 precursor. After transfecting three different colon cancer cell lines with either a miR-137 precursor or a negative control precursor, we carried out BrDU incorporation assays.

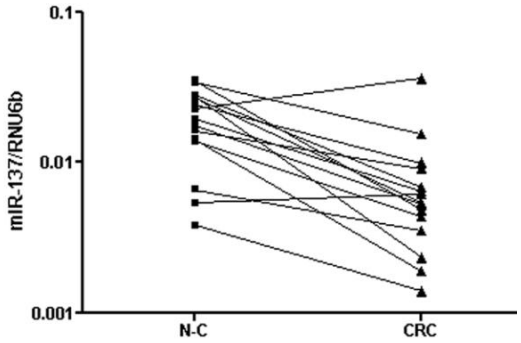

Figure 3: miR-137 CpG island in CRC cell lines

Discussion

In this study, we report that miR-137 promoter methylation is an early event in colorectal carcinogenesis, which results in the epigenetic silencing of miR-137. This miRNA is constitutively expressed in the healthy colonic epithelium and is silenced in malignant tissues, according to in situ hybridization analysis. We were able to pinpoint a number of potential targets of miR-137 using gene expression analysis and in silico prediction tools, including LSD1, a key component of the epigenetic system and a histone demethylase.

Because the average level of methylation in adenomas was similar to that in CRC specimens, our data suggest that methylation of the miR-137 promoter is an early event in colorectal carcinogenesis. Additionally, miR-137 methylation appears to be tumor-specific because it occurs more frequently in neoplastic tissues and its methylation in healthy colonic mucosa did not rise with age.

More intriguingly, we discovered that the histologically normal colorectal mucosa from CRC patients had a higher average level of methylation than the normal mucosa from non-tumor patients, which is consistent with the epigenetic field defect proposed for CRC. This phenomenon is explained by the idea that sporadic CRCs originate from a region of cells that exhibits a "field defect.". Only two prior studies have suggested that miRNA methylation may have analogous manifestation in the gastrointestinal tract, despite the fact that DNA methylation of numerous genes has been proposed as a major contributor to the field defect hypothesis.

The Grady et al. found that 6 out of 9 adenomas and 56% of the normal-appearing colonic mucosa from CRC patients had methylation of miR-342, an intronic miRNA encoded within the EVL gene. In cases of stomach cancer, Ando et al.

Recently, it was discovered that the pattern of miR-124a1-3 methylation in normal gastric mucosa from tumor and healthy subjects is similar. We must note that tissue samples for healthy subjects and CRC patients' normal colonic mucosa were sourced from two distinct populations in Japan and Spain, respectively. Because of this, it may be necessary to conduct additional research to establish with certainty the field defect related to miR-137 methylation in the colon. The differences in miR-137 methylation in these tissues may also be explained by other factors, such as ethnicity or other environmental factors.

Our results for cancer-specificity, the high level of methylation in colonic adenomas, and the potential field defect feature suggest that the methylation status of miR-137 may be used as a potential non-invasive biomarker for CRC. It is unclear which particular cell types within the colon express different miRNAs, despite the fact that numerous studies have shown that the miRNA expression profiles in CRC are significantly different from those in the healthy colonic mucosa.

Since miRNAs are known to play different roles in different cell types and can act as either a tumor suppressor or an oncogene depending on the tissue and cell type, elucidating this aspect is crucial to our understanding of the biological function of miRNAs in carcinogenesis. MiRNA detection using in situ hybridization in FFPE tissues has recently been very successful for various cancers.

We discovered that miR-137 is preferentially expressed in the epithelial cells of healthy colonic mucosa, whereas no miR-137 expression was found in any of the adenomatous polyps and CRC tissues. These results are significant and are in line with our gene-expression data, which revealed downregulation of miR-137 in CRC compared to healthy mucosa.

Given the evidence that miR-137 is frequently silenced in CRC cell lines and CRC tissues as a result of promoter methylation, we carried out functional studies to investigate the potential tumor suppressor properties of miR-137 in vitro. In HCT116 and RKO cells, two cell lines with lower constitutive miR-137 expression, we found that miR-137 restoration did, in fact, result in a significant reduction in proliferation.

Our findings imply that miR-137 might function in CRC as a tumor suppressor miRNA, but more research is required. The discovery of miRNA's gene targets is a crucial and difficult step in understanding miRNA function.

Our understanding of the biological role of miRNAs is limited by the absence of accurate and precise methods for target identification. The most popular method, computational methods, rely on base-pairing between the target and the miRNA seed region (the first 2 to 8 bases of the mature miRNA), but they have problems with specificity because there are typically hundreds or thousands of predicted targets, making it challenging to identify the real targets of a given miRNA.

It is becoming increasingly clear that miRNAs may downregulate a much larger number of transcripts than previously thought, whereas miRNAs were once thought to primarily act through translational inhibition rather than mRNA cleavage. Thus, it has been suggested that gene expression microarray analysis is a practical method for identifying physiologically significant miRNA:mRNA interactions. Through the use of bio-informatic and transcriptomic techniques, potential miR-137 targets were chosen for this study. 32 potential targets were found to have mRNA levels that were downregulated after miR-137 transfection by our rigorous criteria, which helped reduce the false positivity rate.

At least four of the available computational-based prediction tools also predicted these genes as their targets. It was encouraging to see that three of the five chosen genes also displayed corresponding downregulation of protein expression after miR-137 transfection, demonstrating the effectiveness of this tactic, according to our analysis of protein expression.

We discovered an intriguing relationship between miR-137 and the histone demethylase LSD1. LSD1 is a member of a new class of histone demethylating enzymes that, in addition to demethylating H3K4 and H3K9, are crucial for the upkeep of overall DNA methylation through the demethylation of a non-histone substrate, DNMT1.

This is accomplished by increasing the stability of the substrate. Since DNMT1 is expressed more frequently in many human cancers, it is

conceivable that LSD1, which is frequently upregulated in cancer cells, may contribute to this epigenetic flaw. LSD1 is involved in maintaining the undifferenciated phenotype and its function is inhibited when it is overexpressed, as has been shown to occur in neuroblastoma and prostate cancer.

This claim is supported by our discovery of an inverse correlation between miR-137 and LSD1 in CRC cell lines. We then used luciferase experiments to confirm this functional interaction. Future research will elucidate LSD1's additional functions in CRC carcinogenesis and its connection to regulating the expression of miR-137 because it appears to be a key player in the epigenetic machinery and may play a role in cancer therapy.

Conclusion

In conclusion, this study first explains that miR-137 acts as a tumor suppressor in the colon, is frequently silenced in CRC through promoter hypermethylation, and its restoration inhibits cell proliferation in vitro. The use of miR-137 methylation as a CRC biomarker is very promising because it is an early-event in the colon. LSD1 in CRC is one of many potential mRNA targets of miR-137 that we have discovered, and this new information demonstrates how miRNAs interact with other elements of the epigenetic machinery. These results suggest that miR-137 precursors may one day be useful therapeutically for CRC patients.

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